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<p>During the past five years, our laboratory has investigated the secretory IgA memory response to shigella are taken up for antigen processing by the gut-associated lymphoid tissues. The uptake by M cells overlying lymphoid follicles in the gut may serve as a double-edged sword. Whereas this is the initial step in processing of the shigella antigens to develop a mucosal immune response, it is also a portal of entry through which pathogenic shigella prefer to pass and where they can replicate creating the ulcerations characteristic of human dysentery. The cellular basis for the secretory IgA response has been documented in the rabbit model. The role of T lymphocytes from GALT to help the B lymphocyte production of IgA against enterically primed antigens has been shown conclusively. It is especially interesting that these T helper cells can augment the response of B lymphocytes in the peripheral blood (a site not previously studied for this effect despite its availability). Further, we have established that a secretory IgA response against Shiga toxin can be elicited in our Thiry-Vella loop model in rabbits. By using the antibodies</p>			
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from these studies in both in vivo and in vitro evaluations, we have demonstrated that the IgA anti-Shiga toxin can protect against the toxic effects of that molecule. Much of the secretory IgA immune response against Shiga toxin can be elicited in the A and B subunits of Shiga toxin when a crude preparation containing many other antigens is used to immunize rabbits intralooop. A mouse model for following the secretory IgA memory response to shigella antigens has been developed. Using Shiga toxin in this model, we have shown it to be a particularly strong mucosal antigen which may be able to serve as an adjuvant to enhance the secretory IgA response against other antigens. The mouse model system will allow us to take advantage of the large library of monoclonal reagents and inbred strains to further our understanding of the basis for the secretory IgA memory response. Such an understanding is key to efficient development of future vaccines against shigella and other enteropathogens. Our laboratory has completed its studies of the mucosal immune response to shiga toxin and the possible adjuvanticity of shiga toxin for unrelated co-administered protein antigens. The mouse orogastric lavage technique is a useful system in which to examine the immune response in the gut to Shiga toxin. We have found Shiga toxin to be a powerful oral immunogen which does not exert an adjuvant effect on an immunologically unrelated protein. This in contrast to Cholera toxin which is both a powerful oral immunogen and a powerful mucosal immune adjuvant. Lastly, we have evaluated the use of E. coli LT-B as either a covalent carrier or a combined mucosal adjuvant for S. flexneri LPS. While a modest increase in the secretory IgA response against LPS was found when the LT-B was mixed with the LPS, the response was too small to be significant statistically. The present studies, then, have provided a likely pathogenic mechanism for dysentery, established the cellular basis for a mucosal memory response, described the immunogenicity of shiga toxin for the secretory IgA response, and ruled out shiga toxin as a mucosal adjuvant.

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INTRODUCTION

In the present studies, our laboratory has established experimental methods in both mice and rabbits to elicit a strong secretory IgA memory response in intestinal secretions against antigens present on or secreted by *Shigella*. Our chronically isolated ileal (Thiry-Vella) loop model in rabbits has been used to follow the secretory IgA response (1). We demonstrated previously the existence of a secretory IgA memory response against *Shigella* lipopolysaccharide antigens in intestinal secretions following appropriate priming (2). By using the variety of strains of *Shigella flexneri* produced in the laboratory of Dr. Samuel B. Formal at the Walter Reed Army Institute of Research, our studies established that secretory IgA memory responses could be elicited by many different types of *Shigella* (3,4). The present studies establish the role of T lymphocytes in developing the mucosal IgA memory response to shigella antigens, demonstrate the strong immunogenicity of purified shiga toxin, but report that shiga toxin lacks the potent adjuvanticity of cholera toxin, and established the M cell as a pivotal point in the invasion of *S. flexneri* (5-11). Lastly, in collaboration with Dr. John Clements at Tulane University and Dr. Formal we have found that cloned B-subunit from heat labile *E. coli* toxin has only a modest adjuvant effect for mucosal immunity against lipopolysaccharide (LPS).

The early studies with the Thiry-Vella loop model used the hybrid strain of *S. flexneri* and *Escherichia coli* (*Shigella X16*) to establish that an IgA response could be elicited to nonpathogenic antigens and to determine the different methods of immunization required to stimulate the mucosal versus the systemic immune response. The *Shigella X16* strain invades the surface epithelium but does not reproduce once it is within host tissues, therefore, no ulceration is produced. By giving this bacterium directly into isolated intestinal loops, a strong local IgA response was produced (5). Similar responses were found with the invasive strain M4243 (which does cause ulceration) and with a noninvasive strain 2457-0 when applied directly into the isolated ileal loops. The presence of a Peyer's patch locally within the isolated loop and the dosage schedule were other important factors influencing development of the mucosal immune response (5). These early studies proved that when Thiry-Vella loops were stimulated directly with various *Shigella* preparations, secretions collected from those loops would contain considerable antigen-specific secretory IgA but little or no IgG directed against *Shigella*. This was not due to rapid degradation of IgG (which is normally destroyed quickly in intact intestine) since the isolated ileal loops were separated from the proteolytic effects of gastric acid, bile and digestive enzymes including trypsin, pepsin and chymotrypsin. Direct stimulation of the isolated loops by *Shigella* antigens resulted in little or no systemic IgG against *Shigella* unless

the systemic immune response had been previously primed by a parenteral dose of *Shigella*. To elicit IgG in the serum, it was necessary to give the bacteria parenterally; that route resulted in virtually no secretory IgA anti-*Shigella* LPS.

To evaluate the secretory IgA memory response, we immunized rabbits orally with three doses of live or killed *Shigella flexneri* before the Thiry-Vella loops were created. This more natural route of immunization was used to approximate the situation in vaccinating humans. After the animals rested for two months, a chronically isolated ileal loop was created. An oral challenge dose of live *Shigella* was given and secretions from the chronically isolated ileal loops were assayed for the secretory IgA anti-*Shigella* LPS response using an enzyme-linked immunosorbent assay (ELISA). The use of the Thiry-Vella loops as a probe was based on background information about lymphocyte trafficking after intraluminal antigen stimulation for a secretory IgA response. The initial step in stimulation of the secretory IgA response involves phagocytosis of antigens by specialized surface epithelial cells, M cells which are present within the epithelium overlying lymphoid structures throughout the gastrointestinal tract. Through these M cells, antigenic material, microorganisms and soluble proteins are brought into the underlying gut-associated lymphoid tissues (GALT). Once within GALT, these antigens come into contact with precursor B-lymphoblasts which are genetically predisposed to develop secretory IgA response (12). The presence of M cells overlying isolated follicles and Peyer's patches in the small intestine and colon led us to study the role of M cells in the pathogenesis of the focal and patchy ulcerations of the colon and small bowel which are characteristic of dysentery.

The B-lymphocytes that are in GALT come under the influence of specific regulatory cells, originally described as "switch T-cells" by Kawanishi et al. (12). The latter cells encourage B-lymphocytes to alter the phenotype of their surface heavy chain from mu chain to alpha chain. In addition, there are other helper T cells in GALT which help B-lymphocytes mature to IgA secreting plasma cells (13-15). Although the precise mechanism of this switch is unclear, it is known that interleukins 4 and 5 play a role in the process (16,17). Following their stimulation in GALT, these antigen-specific B-lymphoblasts migrate in turn to the mesenteric lymph nodes, the thoracic duct, and eventually lodge in the spleen where they undergo some degree of maturation (18,19). The final site of lodging by these B-lymphocytes may be somewhat influenced by the location of initial antigen stimulation (20). That is, when antigens are applied directly to the respiratory tract, the antigen-specific B-lymphocytes are more likely to return to that site than to the gastrointestinal tract or to mammary secretions. Conversely, when antigen is applied to sites in the gastrointestinal tract antigen-specific B-lymphocytes are

more likely to return to the gastrointestinal tract than to other mucosal surfaces. In our studies of the mucosal memory response, we took advantage of this lymphocyte trafficking to use the chronically isolated ileal loops as a probe for the mucosal memory response. We found that live noninvasive shigella were as effective as virulent invasive strains of *Shigella* at eliciting a secretory IgA memory response against *Shigella* LPS (2-4). These strains did not produce any pathologic lesions in the rabbit intestine.

Five major goals were set for the present studies. 1. examination of the mechanism by which the *Shigella* antigens were initially taken up by the GALT. These ultrastructural studies indicated that avirulent strains of *Shigella* (even heat-killed) were taken up by the follicle-associated epithelial M cells (21). However, virulent strains of *Shigella flexneri* were able to replicate once within this GALT epithelium resulting in destruction of the dome areas and ulceration of the epithelium. These focal lesions may serve as the initial sites where *Shigella* prefer to enter host tissues and spread laterally. The many isolated follicles present in the colon and ileum could serve as preferential sites of entry by this microorganism.

2. examination of the cellular basis of this secretory IgA memory response. We found that by the fourth day following oral rechallenge, sufficient numbers of antigen-specific IgA B-lymphoblasts are present in Peyer's patches and mesenteric lymph nodes that their secreted product in culture can be easily detected by ELISA. These cells were so consistently found in our early studies that we may be able to use their presence to infer establishment of a mucosal memory response.

3. examination of the various specificities of the secretory IgA response to *Shigella* antigens. For this, we used our rabbit loop system to examine the secretory IgA response against Shiga toxin. Extremely strong secretory IgA responses in intestinal secretions were elicited consistently by direct intraloop immunization with Shiga toxin preparations provided by Dr. J. Edward Brown. At the same time, systemic IgG responses are also elicited by this mucosal immunization route. The secretions from this work were used as part of the studies on the functional significance of secretory IgA against shigella antigens (see below). Using Western blotting technique, we examined the heterogeneity of the IgA responses against Shiga toxin. Overall, most antibodies were directed both against the A and B subunits with the A subunit antibody predominating. Relatively weak activity was found against other antigens. The titer of the secretory IgA response to Shiga toxin was higher than that seen against any antigen previously used in our loop model system except for cholera toxin (22). There are similarities between Shiga toxin and cholera toxin. Both are heterodimers composed of A and B subunits; in both the multiple B subunits bind to specific receptors on host epithelium, and in both the single A subunit mediates the toxic effects of the molecule. These observations encourage the hypothesis that both cholera toxin and Shiga toxin

share a common mechanism for activating the secretory IgA response. Since cholera toxin has been shown to serve as an adjuvant to stimulate the mucosal immune response against other antigens co-administered or conjugated to it, we will examine whether secretory IgA against Shiga toxin will also show such an effect. Demonstrating that Shiga toxin is a mucosal adjuvant with a mechanism of action similar to that of cholera toxin, will pave the way to develop mucosal immunity against virtually any antigenic determinant by using one of these two adjuvants as carrier protein. To this end, we have begun establishing a mouse model (see above) which will allow us to examine both the humoral and cellular basis of the secretory IgA response against Shiga toxin.

4. We sought to establish a mouse model to study mucosal immunity to *Shigella* antigens. For the mouse model of the secretory IgA response to Shiga toxin, we have used the Elson technique, previously described with cholera toxin (23). Our studies indicate that a consistent secretory IgA response is elicited in the secretions with as little as 0.1 ml of crude Shiga toxin administered orally. The animals also demonstrate a strong IgG anti-Shiga toxin response in the serum. Relatively little IgM anti-Shiga toxin has been found. Future studies will concentrate on the IgA responses in intestinal secretions. The cellular basis of this response will be easier to study in the mice than in our rabbit model, as many monoclonal antibody preparations specific for mouse helper and suppressor T-lymphocyte populations are available. This will greatly facilitate future studies on the secretory IgA response against these *Shigella* antigens.

5. evaluation of the biologic role of secretory IgA responses against shigella antigens. For these studies, we examined two possible modes of protection. The first involved using ligated loops of intestine to determine whether secretory IgA anti-Shigella LPS from the animals immunized orally with live *Shigella* would alter the uptake of the live bacteria by GALT. For the second, we examined both *in vitro* and *in vivo* model systems to determine whether secretory IgA against Shiga toxin is able to protect from the damaging effects of this molecule in a dose dependent fashion (24). The former studies have not been successful to date, whereas the latter have shown secretory IgA to be most effective in preventing the effects of Shiga toxin. While our studies showed that animals receiving as many as five or six immunizations had a decrease in the number of *Shigella* taken up by the GALT, a large series of studies in which three oral immunizations were used, showed no significant difference in the uptake of *Shigella* by antigen-specific secretory IgA. The current protection models are inadequate to show uptake in a natural situation as they currently require us to use as many as 10^9 bacteria which likely overwhelm the immune response. In the natural infection, it is highly unlikely that numbers anywhere near 10^9 cause the

disease. However, in our experimental models it has not been possible for us to have reproducible uptake of bacteria with fewer microorganisms than this. In the future, by using the RITARD model, we may be able to better approximate the natural infection requiring many fewer microorganisms to provide reproducible bacterial uptake. The studies on the ability of secretory IgA against Shiga toxin to inhibit the toxicity of that molecule have been very successful. Both *in vitro* using the HeLa cell assay and *in vivo*, using acute, ligated loops of intestine in rabbits, IgA anti-shiga toxin has been able to inhibit the effects of Shiga toxin. Future studies will be directed to establishing an effective method to elicit a mucosal memory response to Shiga toxin to determine the duration of the protection achieved.

These studies continue to expand our understanding of the importance of the mucosal immune response in protection against enteropathogens and their toxic products. By exploring the precise modes of stimulation, the types of antigen and adjuvants which will consistently elicit mucosal memory responses and learning the cellular basis for these responses, we will be able to provide effective vaccines against many types of debilitating infectious diseases of mucosal surfaces.

BODY

I. METHODS

In Vitro Culture System. Rabbits were euthanized 1,3,4,5,6 and 10 days after the final boosting dose of *S. flexneri*. Dissection and tissue removal were performed under aseptic conditions. Single cell suspensions of lymphoid cells from the peripheral blood, spleen, mesenteric lymph nodes and Peyer's patches were prepared for tissue culture. Peripheral blood was diluted 1:2 with PBS. 10 ml of whole blood was overlaid onto 3ml of lymphocyte separation medium (Organon/Teknika, Durham, NC) in a 15 ml polystyrene conical tube and centrifuged at 2000 rpm for 25 minutes at room temperature (RT). The cells at the interphase were collected into 50 ml conical tubes, diluted with PBS, washed twice at 1100 rpm, 10 min at RT.

Tissue lymphocyte preparation: After euthanization, the spleen, MLN, and PP were placed immediately into separate sterile petri dishes containing sterile ice cold PBS and RPMI-1640. Because the PP surface is contaminated by luminal bacteria, it was washed in RPMI-1640 containing penicillin (10 U/ml), streptomycin (10ug/ml), and fungizone (.25ug/ml). The tissues were cut into smaller fragments and placed onto a 100 mesh wire screen. The fragments were teased with a 26 g needle and then rinsed with a vigorous stream of PBS forced through an 18 g needle attached to a 60 ml syringe. The dispersed lymphocytes were collected into 50 ml tubes containing cold PBS.

The cell pellet was resuspended in RPMI-1460 with L-glutamine, 10% heat-inactivated fetal calf serum, with antibiotics (see above) and 5×10^{-5} M 2-mercaptoethanol. Viable cells not stained with 0.4% trypan blue were counted using a hemocytometer. Cells were cultured at final concentration of 4×10^6 cells/ml in 24 well culture plates (Costar, Cambridge, MA) and placed in a 37°C incubator with 5% CO₂ and 95% humidified air (Forma Scientific, Marietta, OH).

In Vitro Antigen Stimulation. Single cell suspensions of PP, MLN, SPL, and PB lymphocytes are placed into culture with *S. flexneri* LPS and cholera toxin. LPS is diluted in culture medium (CM) and added to cell cultures at final concentrations of 0.01, 0.1, 1.0, and 10 ug/ml. CT, at final concentrations of .001, .01, .1, and 1.0 ug/ml was added separately. In several experiments the four concentrations of CT were added together with 1.0 or 10.0 ug/ml LPS. These antigens were added to the cell suspensions on the first day of culture. Cell supernatants were collected on days 1 through 7, 14, and 21 of culture. The supernatants were collected using plastic disposable Pasteur pipettes and frozen until assay.

Preparation of Chronically Isolated Ileal Loops in Rabbits. The surgical creation of chronically isolated ileal loops in rabbits has been described in

detail previously (1). Briefly, 3 kg New Zealand white rabbits (specific pathogen free) are anesthetized with xylazine and ketamine. A midline abdominal incision is made and the terminal ileum is identified. 20 cm of ileum containing a Peyer's patch is isolated with its vascular supply intact. Silastic tubing (Dow-Corning) is sewn into each end of the isolated segment. The free ends of the tubing are brought out through the midline incision and are tunnelled subcutaneously to the nape of the neck where they are exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis. The midline incision is closed in two layers. Each day, about 2-4 ml of secretions and mucus that collect in the ileal loops are expelled by injecting 20 ml of air into one of the silastic tubes. Mucus is separated from the clear supernatant by centrifugation. The supernatant is stored at -20°C and assayed for specific immunoglobulin content. Care of the isolated loops requires a subsequent flush with 20 ml of sterile saline to remove adherent mucus. This saline is removed by repeated gentle flushes of air. With proper daily care, > 90% of our rabbits have completed experiments lasting for 2 months.

Mouse Lavage Model for Mucosal Immunity. The mouse model system is an adaptation of one used by Elson for studies of the secretory IgA response to cholera toxin. For these studies, specific pathogen free mice were given 4 oral doses of lavage solution 15 minutes apart. Thirty minutes after the final lavage dose, a single intraperitoneal dose of 0.1mg pilocarpine was given. Pilocarpine encourages the mouse intestine to secrete large volumes of fluid. The mice are placed on wire mesh over beakers containing 3ml of protease inhibitor solution (soybean trypsin inhibitor in 50mM EDTA). Intestinal fluid and feces, and saliva, were collected over 30 minutes. The fluid collected is brought up to 5 ml volume with PBS and vortexed. After centrifuging the fluid at 650 X g for 10 minutes, 10µl of 100mM PMSF in 95% ethanol were added per ml of supernatant. The sample was then centrifuged 27,000 X g at 4°C for 20 minutes and the supernatant was saved. Again 10µl of PMSF in 95% ethanol and 10µl of 1% NaN₃ were added along with 50µl of fetal calf serum to each ml of the final supernatant. Samples were stored at -20°C until time of assay. Blood samples were taken from the animals by retroorbital bleeding.

Enzyme-linked Immunosorbent Assay (ELISA). Microtiter wells are coated with a solution containing the antigen of interest. For studies on Shiga toxin, we use the purified Shiga toxin preparation (see below) and for experiments on Shigella antigens, we have used a Westphal (hot phenol-water) extraction from *Shigella flexneri*. Concentration of the coating antigen is empirically determined with each new preparation of antigen. Standard IgG

and IgA positive and negative samples are run with each new antigen preparation to ensure continuity from one lot to the next. Immediately prior to testing serum samples, loop secretions or mouse intestinal secretions, the antigen solution is removed and wells are washed with a phosphate-buffered saline solution (PBS) containing 0.05% Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated and uncoated wells (the latter control for nonspecific adsorption) for 4 hours. The plates are washed with PT and incubated with either alkaline phosphatase-conjugated sheep anti-rabbit IgA, sheep anti-rabbit IgG (both isotype specific via affinity column purification in our laboratory as previously described (25)), or monoclonal anti-mouse IgG, IgA or IgM conjugated with alkaline phosphatase (Cappel). After a second incubation of 4 hours, the wells are again washed with PT and the substrate reaction is carried out with p-nitrophenyl phosphate in carbonate buffer pH 9.8. The kinetics of the enzyme-substrate reaction are extrapolated to 100 minutes. For the Shiga toxin assays, results are expressed as titer using a standard as previously described (7). Specific IgG and IgA standards are processed on each plate with the unknown fluids as previously described (25). The data are analyzed using the RS1 integrated software system. Data are presented as titers or as geometric means. For each day's results, the variance is expressed together with the mean. These responses are expected to be increased at ST and CT may have similar biologic effects as protein kinase C-activating phorbol esters (26).

The Amplified ELISA for detecting the anti-shigella activity in cell supernatant experiments used the Bethesda Research Laboratories system (Gathersburg, MD). The substrate reaction for this is carried out under a two step reaction system. First the substrate, containing NADP is added (50 ul/well) and incubated for 15 minutes in the RT incubator. Then the amplifier is added at 50 ul/well. The amplifier contains the enzymes diaphorase and alcohol dehydrogenase which act on idonitrotetrazolium violet and ethanol, respectively. A reduction/oxidation cycle ensues with the reduction of the dephosphorylated NADP. Formazan, a dark red dye, is the resulting end-product. These plates are read on a Biotek EL-310 EIA reader (Biotek Instruments, Inc., Winooski, CT). Optical density readings at 490nm were recorded at 3.75, 7.5 and 15 minutes. The anti-S.flexneri LPS IgA or IgG titer of the cell supernatants were determined. A standard curve using four dilutions of anti-S. flexneri LPS IgA or IgG was run on each plate.

Antigen Preparations Used. Two major preparations of *Shigella flexneri* are used in the present studies: 1) *Shigella flexneri* M4243A₁. This strain lacks the 140 megadalton virulence plasmid and is not able to invade the surface epithelium. 2) *Shigella* X16. This is a hybrid of *S. flexneri* and *E. coli* which is able to invade the mucosa, does possess the 140 megadalton

virulence plasmid, but does not persist within the epithelium following uptake *. Shiga toxin antigen preparations include a crude and purified preparation provided by Dr. J. Edward Brown. The crude preparation is a lysate of *Shigella dysenteriae* type 1 which has been passed over a diethylaminoethyl cellulose column. This preparation had 10^{4-5} CD50/ml. The purified preparation, also provided by Dr. Brown, has been affinity- purified as described previously (27). Polyacrylamide gel electrophoresis and Western blotting of this preparation discloses two major bands consisting of the A and B subunits. A minor band, possibly a fragment of the A subunit, has been found on Western blotting of this preparation with specific antibodies made against the crude preparation.

In Vivo Assay for Uptake of Shigella by Follicle-Associated Epithelium and Villi. To determine the relationship between the virulence of the microorganism and the uptake of the bacteria by the follicle-associated epithelium, an *in vivo* assay procedure was employed. Isolated acute ileal loops 10 cm in length were created in specific pathogen free New Zealand bred white rabbits. A single dose containing 2×10^9 *Shigella flexneri* was injected into this acute loop. At 90 minutes and at 18 hours, these loops were removed and samples were fixed for histologic investigation by electron microscopy and light microscopy. For light microscopy, the sections were fixed in absolute ethanol and stained with Giemsa. For each time period, at least 10 sections of Peyer's patch and adjacent villi were examined for attachment and uptake of the Shigella. Histologically, these sections were divided into 2 areas: 1) the follicle-associated epithelium overlying the dome areas in Peyer's patches (known to be enriched in "M" cells); 2) villi which were outside of the Peyer's patch area. Evaluation was performed using oil immersion light microscopy. Since the normal flora of the rabbit ileum contains $< 10^4$ microorganisms, for statistical purposes, $< 0.01\%$ of the flora visualized were from other microorganisms. Further, the Shigella have a characteristic size and shape which under the circumstances of the this study were readily recognizable. The Bioquant Biometrics Image Analyzer (Nashville, Tennessee) with an IBM computer was used to measure the actual length in millimeters of the lining epithelium over the villi and over the dome regions of the Peyer's patches. The average of 100 fields for dome and villus areas from representative rabbits was calculated. This allowed us to express data as bacteria/mm² of surface epithelium. Therefore, a direct relationship of villus surface area to follicle-associated epithelium surface area was established, allowing for comparisons. Electron microscopy was performed on some sections demonstrating the characteristic rod-shaped structure and typical "M" cell location.

Electron Microscopy. Tissues for study were minced to approximately 1 mm³ and fixed in 3% glutaraldehyde-formaldehyde in 0.1 M cacodylate buffer, pH 7.3. The samples were postfixed in 2% osmium tetroxide. After staining en block with 2% uranyl acetate, tissues were dehydrated in alcohol and embedded in epon. One micron thick sections were cut and stained with toluidine blue and examined for uptake of *Shigella*. Follicle-associated epithelial cells which contained *Shigella* were identified and thin sections (approximately 800 Angstrom's thick) were cut from these areas on a Porter-Blum MT-2 ultramicrotome. These thin sections were stained with lead citrate and examined with a Zeiss 109 transmission electron microscope. Photomicrographs were taken of the characteristic rod-shaped bacilli in the M cells.

Acute Loop Protection Studies. New Zealand white rabbits (specific pathogen free) are anesthetized with xylazine and ketamine. A midline abdominal incision is made and the terminal ileum is identified. A series of 5 cm segments from the mid-jejunum to the mid-ileum were created. Double 4.0 silk ligatures were placed between each segment to prevent leakage from one segment to another. Solutions to be tested for toxin or antitoxin activity were injected into the loops in the doses indicated. The midline incision was closed in two layers and the animals were allowed to rest for 18 hours. At time of sacrifice, the fluid contents of the loops were measured.

Cytotoxicity Assay. Shiga toxin activity was determined by examining the extent of HeLa cell damage by a previously described assay (28). Briefly, HeLa cell monolayers were grown in 96 well microtiter plates. For the assay, a standard crude toxin lysate of *S. dysenteriae* was incubated with serial dilutions of loop fluids for 30 minutes at room temperature. This mixture was placed onto the HeLa cell monolayer and allowed to incubate overnight at room temperature. The monolayers were then stained with crystal violet dissolved in 50% ethanol-1% sodium dodecyl sulfate, and the O.D. 620 nm was determined for each well. The dye remaining in each well correlates with the percentage of cell remaining adherent to the microtiter dishes. O.D. 620 nm of wells containing the standard toxin alone were averaged and that value plus two standard deviations was defined as the end point titer of loop fluids for neutralization of the cytotoxicity of the toxin preparation. All dilutions of loop fluid which gave an O.D. 620 in the assay greater than this value were scored as positive.

Flow Cytometry for Lymphocyte Separations. PP lymphocytes from rabbits immunized with 4 peroral doses of antigen were sampled four days

after the fourth boosting dose. After overnight culture, the PP cells were stained with FITC-conjugated F(ab')₂ goat anti-rabbit IgA, IgM, and IgG (#AQ132F, Chemicon International, Inc., Temecula, CA) for 30 minutes in an ice water bath. After three washes in ice cold PBS containing 1% FCS, antibiotics (see above) and were resuspended at 5×10^6 cells/ml and stored on ice.

An EPICS 751 flow cytometer equipped with an argon laser set at 488 nm was used for cell sorting (Coulter Electronics, EPICS Division, Hialeah, FL). The lymphocyte population was identified and gated on forward light scatter to exclude debris and dead cells. Gating on green fluorescence was accomplished by expanding the green signal to create an exaggerated brightly positive peak and a dimmer negative peak. The right sort gate on the Ig positive peak was set narrowly. The left sort gate was also set tightly from the right slope to the origin. This left several channels between the peaks which resulted in unsorted and therefore wasted cells. This strategy was used to minimize the error of contaminating the Ig+ and Ig- cells and to improve purity of the populations. After sorting, the cells were centrifuged and resuspended in CM at 1×10^6 cells/ml. A viable count was done. The Ig- cells were restained and the purity was confirmed by flow cytometry and independent fluorescent microscopy.

Immunodetection on Nitrocellulose Blots. Samples are subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli (29). The initial gel contains 12.6% acrylamide with a 3% acrylamide stacking gel. Molecular weight markers are applied to the gel as well as the sample of interest. Electrophoretic transfer of proteins is carried out as described by Towbin et al. (30) and Burnette (31). Proteins are electrophoretically transferred to nitrocellulose paper for 2.5 hours at constant voltage (60 V). After transfer, reactive sites remaining on the nitrocellulose are blocked by soaking the paper in tris-buffered saline containing .05% Tween 20 and 3% gelatin. This paper is reacted for 1 hour at 37°C with strips coated with either serum or loop fluid with reactivity to Shiga toxin. Following a gentle wash procedure, alkaline phosphatase-conjugated antibodies monospecific for rabbit IgG or IgA are added for 1 hour at 37°C. The substrate reaction allows visualization of the specific bands.

Preparation of cloned B-subunit-LPS conjugates. A series of experiments were performed in which different molar ratios of LPS and LT-B (provided by John Clements (Tulane University)) were joined using the Avrameas glutaraldehyde coupling technique. Once coupling was complete, the conjugates were separated using Sephacryl S-300 molecular sieve

chromatography, and the earliest eluting peak was retained, with free LT-B eluting later. The degree of coupling was determined by the incorporation of ^{125}I -radiolabeled LT-B into the void volume fraction. The resulting conjugate was dialyzed, concentrated in a stirred cell, and tested by immunoassay for the presence of BM1 binding capacity, the presence of LT-B antigenic determinants, and the expression of LPS epitopes on the BM1-bound conjugates. The final preparation (LPS-LTB-5) demonstrated excellent binding characteristics in all three assays when compared to control preparations of each.

II. Results and Discussion

A. Studies on the Initial Antigen Processing of Different Shigella

Preparations by the Intestine. The first goal of the present studies was to complete our examination of the initial uptake of different preparations of *Shigella flexneri* by the intestine. In the previous study period, we reported results of the uptake of live bacteria; now, the studies using heat-killed bacteria have been completed. With this work, we sought to determine how *S. flexneri* were taken up by the surface M cells which overlie lymphoid follicles in the gut. This initial uptake is important for development of the mucosal immune response to the Shigella antigens.

For these studies, segments of intestine were isolated as outlined in the methods section. Each group contained five rabbits (Table 1) to ensure reproducibility of the model. The bacteria listed in Table 1 were injected directly into each loop at time zero and at 30 minutes, 90 minutes and 18 hours; acute loops were removed and examined by both electron and light microscopy.

Table 1. Strains of Shigella flexneri Used
for M Cell Uptake Studies in Rabbit Loops

<u>Rabbit Group</u>	<u>S. flexneri strain</u>	<u>Plasmid</u> ¹	<u>Ulcer</u> ²	<u>M-Cell</u> ³	<u>Sereny</u> ⁴
1	M4243	+	+	+	+
2	X16	+	-	+	-
3	2457-O	+	-	+	-
4	M4243A1	-	-	+	-
5	Heat-Killed M4243	+	-	+	-

1. Presence of 140 megadalton virulence plasmid.
2. Mucosal ulceration produced in acute loops.
3. Uptake by M-cell demonstrated ultrastructurally.
4. Virulence demonstrated by Sereny test in guinea pigs.

For light microscopy, frozen sections were cut at 4 microns and stained with Giemsa. Ten sections were cut from each loop, for each time period. Therefore, 50 slides for each strain of bacteria, for each time period, were examined. All the slides were coded so that the observer would not know the time point or the particular animal or strain of shigella being examined. The

number of shigella within both the follicle-associated epithelium overlying Peyer's patches and the villus epithelium were counted.

To determine the number of bacteria per unit area, the specimens were standardized using a Bioquant image analyzer. This allowed us to express our results as number of bacteria taken-up per micron of epithelium. It also allowed us to correlate the uptake over isolated follicles with that over surface epithelium. This work has indicated that heat-killed *Shigella* were taken-up to a similar extent as that of nonpathogenic strains of shigella. On average, 1.6 bacteria/mm² of surface epithelium were found in the follicle-associated epithelium, while ten-fold fewer bacteria were present in the adjacent villus epithelium (5). Therefore, the follicle-associated epithelium preferentially ingests both live and killed shigella. No evidence of ulceration was seen in any of the acute loops given the heat-killed shigella (5).

The presence of the bacteria in M cells was confirmed by examining electron microscopic fields of both villus and follicle-associated epithelium. Because light microscopy had shown that the major uptake of *Shigella* had occurred by 90 minutes, samples from this time period were examined. Denatured (heat-killed) *Shigella* were found within phagocytic vesicles of M cells in the follicle-associated epithelium (5). The tissue damage at the 18 hour time period was correlated with the degree of uptake by the M cells in the follicle-associated epithelium (5). All of the rabbits given pathogenic *S. flexneri* M4243 had ulcerations of isolated follicles and Peyer's patch follicles at 18 hours. In marked contrast, none of the animals given nonpathogenic or heat-killed *Shigella* had evidence of ulceration or even microulceration at this time.

In the acute loops given pathogenic *S. flexneri* M4243, evidence of damage to the villus epithelium was observed, but, no ulcerations were seen. There was mucus depletion and occasional focal acute inflammation. These findings indicated that *S. flexneri* is initially engulfed by the M cells wherein they multiply and eventually result in focal ulceration. Both nonpathogenic strains and heat-killed strains are processed by the M cells, which may explain the ability of these nonpathogenic strains to elicit a mucosal immune response against the *S. flexneri* LPS antigens. It is not clear why the heat-killed strain, which is taken-up in similar numbers as the live nonpathogenic strains, has proven to be incapable of priming animals for a mucosal memory response in our previous studies (2,3). It may be that the heat treatment damages antigens necessary for the appropriate stimulation of mucosal immunity.

B. Location of Shigella Antigen-specific Lymphocytes following Oral and Parenteral Priming. In the first part of this contract, we wished to explore both the native production of IgA anti-Shigella LPS by these cells and the effect of common mitogens such as PHA and PWM on this response. Our previous project had established the optimal concentrations of Con A and PWM to use in these studies. Previous studies had shown that following three oral doses of the noninvasive *S. flexneri* M4243A1 (lacking the 140 megadalton virulence plasmid), animals were primed for a mucosal memory response (32). The rabbits were then allowed to rest for 60 days. A single oral challenge dose with the same bacteria was given, and on days 1, 3, 4, 5, 6, and 10 following this, the rabbits were sacrificed and the Peyer's patches, mesenteric lymph nodes, and spleen were separated into single cell suspensions. These suspensions were grown in tissue culture with or without concanavalin A or pokeweed mitogen (using the previously determined optimal mitogenic doses). These cultures were maintained for as long as 3 weeks with samples of the supernatants assayed at various days to determine the specific immunoglobulin production. In addition, a procedure was set up on the RS1 software system for handling the large volume of data which was generated by these studies.

By day 3 following oral challenge with the live *S. flexneri* M4243A1, the lymphocytes from the Peyer's patches and the mesenteric lymph nodes contained considerable amounts of IgA anti-Shigella LPS (10). Only lymphocytes from the spleen were found to produce significant amounts of IgG anti-Shigella LPS. Corresponding cells from control rabbits given only the single oral challenge prior to sacrifice produced virtually no IgA or IgG directed against Shigella LPS in cultures of the Peyer's patches, mesenteric lymph nodes, spleen or peripheral blood.

The mitogens gave inconsistent results in our studies. In some rabbits, the mitogens gave an increase in the IgA response, such as the day 10 response from the Peyer's patches. However, sometimes, the mitogens gave a weaker response such as the day 6 PWM response for the Peyer's patch cells. In one group of rabbits, we performed a mitogen dose response study for Con A and PWM on the specific IgA responses of their cell populations. Assays of cell supernatants from cultures receiving doses of Con A from 0.1 to 1.0 µg/ml, showed no specific pattern of reactivity. On day 14 in culture, the greatest anti-Shigella LPS response was seen with 5 µg/ml. We believe that the mitogens are probably not having a significant effect on the system and the variation we see is just reflecting the heterogeneity of the lymphoid population itself. Therefore, in the second half of the contract, we have decided to eliminate mitogens from future cultures in favor of having more cells for replicate cultures. This will allow us to better estimate the variation between cultures in an individual rabbit.

In the studies performed during the second half of the contract period, we were seeking to determine whether lymphocytes present in various sites, Peyer's patches, peripheral blood, spleen, and mesenteric lymph nodes would contain B lymphocytes capable of producing an IgA response against the apparent LPS. We analyzed cell supernatants of cultured lymphocytes from rabbits which were perorally immunized with two strains of Shigella flexneri: M4243A₁ or 2457-0. These New Zealand white rabbits were immunized with a live, 24 hour culture of either strain growing in brain heart infusion. Three immunizations were given, one week apart. After a 60 day period, a booster dose was given and the rabbits were dissected four days later. Peripheral blood, spleen, Peyer's patches, and mesenteric lymph nodes were separated as described in our grant. These lymphocytes were processed in culture with the addition of cholera toxin or lipopolysaccharide to determine whether these would have an influence on the maturation or development of IgA secreting plasma cells against Shigella flexneri. New Zealand white rabbits were given a live, 24 hour culture of either strain growing in BHI. After three oral immunizations were given weekly, the animals were allowed to rest for 60 days. Then, lymphocytes from the above sites were separated and grown in tissue culture. Cholera toxin and LPS are immunomodulatory molecules for the humoral immune response. LPS is known to cause a polyclonal B cell proliferation and cholera toxin has been shown in our laboratory and others to enhance the initial (primary) mucosal immune response against co-administered antigens. To determine the effects of these immunomodulatory molecules on the mucosal memory response, cholera toxin and/or LPS in graded doses were added to the lymphocyte cultures. Supernatants from these cultures have now been assayed for specific IgG and IgA antibodies. The data from these studies indicates that a vigorous IgA response is usually found in the Peyer's patch and lymph node mononuclear cell cultures (tables 2 and 3). The spleen and the peripheral blood rarely contain the lymphocytes capable of producing IgA against Shigella flexneri LPS. The cholera toxin added in doses from 0.1 mg/ml to 10 mg/ml had no significant enhancing effect on the ability of the cultured lymphocytes to produce an IgA response. Further, the lipopolysaccharide added at 100 mg/ml did not promote the production of IgA against Shigella flexneri LPS. These studies indicate that the memory B lymphocytes present within the gut-associated lymphoid tissues are likely fully differentiated and do not proliferate to known mucosal immunomodulatory molecules.

Table 2. Summary of Anti-S. flexneri LPS IgA Production by 14-day Cultured Lymphocytes

	<u>Days post-Boost</u>					
<u>Peyer's patch</u>	<u>1</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>10</u>
mean	.4	4.2	4.4	3..7	2.3	3.0
Std	.4	1.1	1.9	2.9	.3	1.7
n	2	3	10	3	2	5
<u>MLN</u>						
mean	0	1.9	1.8	1.7	0.5	1.5
Std	0	1.8	1.8	1.4	0.7	1.2
n	3	3	11	3	3	5
<u>SPL</u>						
mean	1.0	3.5	1.7	0.2	0	2.6
Std	0.8	2.3	1.6	0.2	0	1.5
n	3	3	11	3	3	5
<u>PB</u>						
mean	0	1.5	0.5	1.4	1.0	0.7
Std	0	0	0..5	1.0	1.0	0.9
n	2	1	9	2	1	5

Value shown are means of pooled titers expressed as log₂.

In order to study the regulatory events surrounding the secretory IgA response to lipopolysaccharide, we purified T-cell population from rabbit Peyer's patches. For our initial studies, we used complement-mediated cytotoxicity and the anti-rabbit B-cell antibody characterized in the literature as RABELA. In one rabbit which had been fully immunized with M4243A1 and dissected four days post-boost, RABELA positive Peyer's patch cells were successfully killed, leaving a populationulation of mostly T-cells and macrophages. These cells were placed in culture with spleen and mesenteric lymph node cells from the same rabbit. The T and B cell populations proved difficult to separate in the rabbit. We tried to use commercially available antibodies specific for rabbit B and T cells (RABELA and RATELA, see above) in a complement-mediated cytotoxicity assay. These studies were unsuccessful.

Therefore, we developed a method using antibodies to rabbit surface immunoglobulin combined with flow cytometry and have succeeded in separating the B and T lymphocyte populations to 98% purity. For these studies, the rabbit Peyer's patch lymphocytes were used exclusively. On day four post-boost the rabbits were sacrificed and dissected. After teasing the cells apart, the mononuclear populations were placed on lymphocyte separation media and centrifuged. The lymphocyte layer was stained with a

fluorescence\ (FITC) - conjugated goat anti-rabbit IgG, IgA, IgM antibody or FITC-conjugated goat anti-rabbit IgG. Cell sorting for FITC positive and FITC negative cells was performed by flow cytometry the sorted cells were then examined by direct fluorescence microscopy for the presence of surface immunoglobulin cells. The B cell population was 98% positive for surface immunoglobulin cells and the non-B (T) cell population contained <2% surface immunoglobulin cells. These cells were placed separately into tissue culture with purified mesenteric lymph node, Peyer's patch, spleen, or peripheral blood lymphocytes from the same animal. After 14 days in culture, the supernatants were ready for assay.

Table 3. Rabbits Sampled Five Days Post-boost, Anti-*S. flexneri* LPS IgA Response in Peyer's Patch Cultured Lymphocytes.

<u>Culture</u> <u>Well</u>	<u>LA-15</u>	<u>LA-25</u>	<u>LA-35</u>
1	0	3	6
2	0	4	7
3	0	5	8
Mean	0	4	7

LA = individual rabbit number

Values are titers expressed as log₂ of individual cultured wells.

We found that the enriched Peyer's patch surface immunoglobulin negative population (T-enriched) can induce purified lymphocytes from the peripheral blood and spleen to produce specific IgA anti-*Shigella flexneri* LPS. In both rabbits, the Peyer's patch surface immunoglobulin negative lymphocytes caused a positive specific IgA response in the supernatants of cultured spleen and peripheral blood lymphocytes. In one rabbit, the Peyer's patch surface immunoglobulin negative lymphocytes raised the titer of splenic and peripheral blood lymphocytes from <1 to log base 2. In the other rabbit titers as high as 3 were seen (See Table 4).

**Table 4. Anti-S.flexneri LPS IgA Production of Sorted PP Lymphocytes
Cocultured with Whole Cell Preparations.**

Cell ratio 1:1

<u>Rabbit</u>	<u>None</u>	<u>Cells Added:</u>	
		<u>T lymphocytes</u>	<u>B Lymphocytes</u>
LA-41			
PP	<1,2,4	1	ND
MLN	<1,<1	<1,2	<1,2,4
SPL	<1,<1	3,3	<1,<1,4
PB	<1,<1	1,5	<1,2
LA-44			
PP	<1,<1,<1,<1	<1	<1,<1
MLN	<1,<1,<1,<1	<1,<1	<1
SPL	<1,<1,<1,<1	2,2	<1
PB	<1,<1,<1,<1	2,3	<1
LA-45			
PP	<1,<1,<1,<1	<1	<1,<1
MLN	<1,<1,<1,<1	<1,<1	<1
SPL	<1,<1,<1,<1	<1,<1	<1
PB	<1,<1,<1,<1	<1,<1	2
LA-46			
PP	<1,<1,<1,<1	<1,<1	<1,<1
MLN	<1,<1,<1,<1	<1,<1	<1,<1
SPL	<1,<1,<1,<1	<1,<1	<1,<1
PB	<1,<1,<1,<1	2,3	<1,<1

* SPL = Spleen
MLN = Mesenteric Lymph Node
PB = Peripheral Blood
PP = Peyer's Patch

*Anti-S. flexneri LPS IgA expressed as log2 of titer. Individual culture wells from 96 well plates were analyzed separately and are represented. Results from individual rabbits are shown.

Cell ratio 1:1 is the ratio of PP T cell: whole cell preparation, both at 1×10^6 cells/ml.

As stated above, this is an effect that we have not seen in animals given this regiment therefore, the T-cell enriched population from the Peyer's patches is able to enhance the production of IgA from peripheral lymphoid populations. The T-lymphocytes then, appear to be the controlling factor in determining the production of mucosal memory response at distant sites. These Peyer's patch surface immunoglobulin negative lymphocytes, from immunized and boosted rabbits are probably the memory T-cells specific for anti-Shigella flexneri LPS IgA. Furthermore, they are ready to stimulate IgA class switching in susceptible B-lymphocytes located in the spleen and peripheral blood. It is interesting that in both rabbits studied the enriched Peyer's patch T-lymphocytes caused IgA production of B-cells in the peripheral blood and spleen but not within the mesenteric lymph node tissues.

With this methodology complete, we used two concentrations of T-cell to target cells in recognition of the fact that ratio of T to B lymphocytes can be crucial in achieving an appropriate immune response. With a 1:1 ratio of added T cells:test cells (spleen, mesenteric lymph nodes, Peyer's patches, or peripheral blood lymphocytes), a highly significant helper effect was seen in the peripheral blood and spleen for an IgA anti-Shigella LPS response. Such a response from peripheral blood and splenic B lymphocytes has not been seen previously by us or others (Table 5). It indicates that B lymphocytes capable of an IgA response to enterically-administered antigens are present at these peripheral sites and, under appropriate stimulation from specific T-helper lymphocytes, will mature to produce plasma cells secreting IgA against these enterically-primed antigens. This could have considerable significance for eventual development of vaccines against enteric pathogens. No help was seen for the Peyer's patch and mesenteric lymph node B-cell populations. This presumably reflected the fact that these populations already contain sufficient helper T-cells to give a maximal IgA anti-Shigella LPS response. Notably, addition of B-lymphocytes to these populations produced no significant augmentation of the IgA response. The 1:4 concentration of added T cells:test cells do not show a helper effect. Presumably too few helper T cells were present to aid the maturation of the antigen-specific B lymphocytes. These studies indicate that helper T-cells provide a crucial point for regulation of the IgA response at a 1:1 concentration.

Table 5. Anti-S.flexneri LPS IgA Production of Sorted PP Lymphocytes Cocultured with Whole Cell Preparations.

Cell ratio 1:4

<u>Rabbit</u>	<u>None</u>	<u>Cells Added:</u>	
		<u>T</u> <u>lymphocytes</u>	<u>B</u> <u>Lymphocytes</u>
LA-44			
PP	3,3,3,4	1	2,3
MLN	1,1,2,5	1,1	<1
SPL	<1,<1,<1,<1	<1,<1	1
PB	<1,<1,<1,<1	<1,<1	<1
LA-45			
PP	4,4,5,5,	3,4	3
MLN	1,1,1,3	<1,3	2
SPL	<1,<1,<1,<1	<1,<1	1
PB	<1,<1,<1,<1	<1,<1	<1
LA-46			
PP	1,2,2,4	<1,<1	2,2
MLN	<1,<1,<1,<1	<1,<1	<1,<1
SPL	<1,<1,<1,<1	<1,<1	<1,<1
PB	<1,<1,<1,<1	<1,<1	<1,<1

SPL = Spleen

MLN = Mesenteric Lymph Node

PB = Peripheral Blood

PP = Peyer's Patch

*Anti-S. flexneri LPS IgA expressed as log2 of titer. Individual culture wells from 96 well plates were analyzed separately and are represented. Results from individual rabbits are shown.

Cell ratio 1:4 is the ratio of PP T cell: whole cell preparation, both at 1×10^6 cells/ml.

Statistical analysis of anti-S. flexneri LPS IgA production of the sorted cell cultures showed that added PP T cells could enhance the IgA production of SPL and PB at a cell ratio 1:1 (Table 6) over the IgA production of these cell cultured alone. both of these responses were biologically significant because there was a minimum difference of two titers between groups. This resulted in an overall statistically significant ($p=.017$) difference of IgA production between cultures of PP T cells added to SPL cell preparations over that of SPL cell cultures alone. The enhancement of specific IgA production when PP T cells were added to PB lymphocytes was highly significant ($p=0.0002$).

In the experiments of 1:4 cell ratio, a statistically significant suppression of IgA production in the whole cell cultures of PP occurred when PP T cells were added over that of PP whole cells alone ($p=.021$). The 1:4 cell ratio of SPL cell cultures with PP T cells added appeared to show statistical significance ($p=.042$), however, the confidence limits of the Scheffe allowance indicated this not to be a significant value. The biological significance was also lacking in this comparison because there was only one titer difference between the cultures of SPL cells alone and of B cells added (Table 6).

Table 6. Statistical Analysis of Sorted PP Lymphocyte co-cultured with Whole Cell Preparations.

<u>Cell Type</u>	<u>P value</u>	<u>no cells added vs. PP T cells</u>	<u>no cells added vs. PP B cells</u>
Ratio 1:1			
PP	0.0460	0.403	0.269
MLN	0.155	0.642	0.057
SPL	0.055	0.017	0.300
PB	0.001	0.0002	0.198
Ratio 1:4			
PP	0.055	0.021	0.182
MLN	0.738	0.545	0.527
SPL	0.101	1.000	0.042
PB	0.438	0.274	0.342

*Sorted PP lymphocytes were co-cultured with whole cell preparations of PP, MLN, SPL, and PB. Values expressed are P values based on F ratios of anti-S.flexneri LPS IgA production of cultured cell populations. Titer values were pooled from all rabbits for each culture type for analysis by the Scheffe allowance.

P values <0.05 indicate enhancement of the IgA response.

P values >0.05 indicate suppression of the IgA response.

These studies provide insight into the cellular aspects of the mucosal memory response to *S. flexneri*. The distribution of anti-S. flexneri LPS IgA producing cells in rabbits which have been orally primed and boosted with live avirulent *S. flexneri* M4243A1 is demonstrated. This work shows that specific antibody-producing cells can be found in the PP, MLN, SPL and PB following oral priming and boosting. These findings suggest that migration of specific antigen-reactive cells occurs from the gut, the site of immunization, to sites within GALT (MLN), and in the systemic immune compartment (SPL

and PB). These findings also show that the greatest distribution of anti-S. flexneri LPS IgA producing cells occurs in the PP. The PP cultured lymphocytes produced specific IgA in greater amounts than cultured cells from the MLN, SPL, and PB. This provides evidence for the cellular distribution of the mucosal immune response in rabbits orally immunized with avirulent S. flexneri M4243A1.

Very few specific anti-S. flexneri LPS IgG producing cells were found in the PP, SPL, MLN, and PB. This may reflect oral tolerance. PP lymphocytes from rabbits which had been fully primed and boosted were separated into T and B cell populations and cultured with whole lymphocyte preparations from the PP, MLN, SPL, and PB. Shown here is the ability of PP T cells to enhance antigen specific IgA responses in both the SPL and PB. Cultured PB cells with PP T cells added showed an enhanced IgA response. SPL cell cultures also demonstrated an increase in specific IgA production with the addition of PP T cells. Cultured lymphocytes from these organs generally produce very low amounts of anti-S. flexneri IgA. This provides further insight that regulatory T cells reside in GALT and can preferentially select and help in the differentiation and expansion of antigen specific B cells in infections involving mucosal surfaces. Along with the evidence of oral tolerance, it is likely that the anti-Shigella mucosal immune response is under the control of T cells from GALT. These studies thus enhance our understanding of cell regulatory properties in the mucosal immune system and how they influence infections at mucosal surfaces. The information from these studies should be applied to the logical creation of new vaccines against infections which occur at mucosal surfaces.

C. Heterogeneity of Mucosal and Systemic anti-Shigella Responses. In the past, we have exclusively used ELISA on Shigella LPS (Westphal preparations) to study the secretory IgA responses. This technique limits our ability to have a detailed understanding of the key antigens involved in eliciting a strong secretory IgA response. Therefore, in the present studies on anti-Shigella LPS and anti-Shiga toxin responses, we will be using the Western blot assay to better determine key antigenic determinants which may be responsible for the secretory IgA responses seen.

During the first half of the contract period, we developed the SDS-polyacrylamide gel electrophoresis and Western blot techniques for use with our model systems. We have developed our own mixture of molecular weight standards for the gels which include: lysozyme, beta-lactoglobulin, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase b. These cover the range of molecular weights from 14,000 to 96,000. The bacterial cell antigen preparations are made by boiling the cells for 5 minutes in phosphate buffer containing 1% SDS, 10% glycerol and 5 μ M PMSF. We

have used this assay in recently completed studies on the IgA response to Shiga toxin. This response is mainly against the A and B subunits, although other reactivities are seen. The prominence of the reaction against the A and B subunits suggests that Shiga toxin may be a particularly strong mucosal stimulant. As discussed in the next section, the strength of the mucosal immune response to Shiga toxin is matched only by previous studies with cholera toxin in our laboratory.

D. Mouse Model for Mucosal Immunity to Shigella Antigens. While the chronically isolated ileal loop model in rabbits has allowed us to characterize many of the important variables involved in stimulating the mucosal memory response to *S. flexneri*, the outbred nature of the rabbit has limited the depth to which we could study the observed reactions. We are limited in terms of available reagents, genetic details of immune responsiveness and in the heterogeneity of the responses elicited. Furthermore, the lack of histocompatibility prevents cross culture experiments with our available *in vitro* systems. Therefore we have established a mouse model for examining the secretory IgA response to Shigella antigens. We have chosen Shiga toxin (ST) to study, as little is known about the mucosal immune response to this molecule and our studies in rabbits indicate that it may be a strong mucosal immunogen. Further, the antibody response to this antigen may protect against its toxic effects (see next section).

In the present studies, we examined the role of cholera toxin (CT) and ST to serve as both immunogens for the mucosal immune response and as adjuvants to stimulate a secretory IgA response against relatively poor mucosal immunogens. We first sought to determine the dose response curve using purified ST preparations. For these studies, we used the mouse lavage model system. Specific pathogen free C57Bl6/J mice (Jackson Laboratories, Bar Harbor, ME) were given intragastric doses of from .01 to 50 mg of purified ST (provided by Drs. A. Donohue-Rolfe and G.T. Keusch) on days 0,7,14, and 21. Secretions were collected by administering 4 oral doses of lavage solution 15 minutes apart. Thirty minutes after the final lavage dose, a single intraperitoneal dose of 0.1mg pilocarpine was given. Pilocarpine encourages the mouse intestine to secrete large volumes of fluid. The mice were placed on wire mesh over beakers containing 3ml of protease inhibitor solution (soybean trypsin inhibitor in 50mM EDTA). Intestinal fluid and feces, and saliva, were collected over 30 minutes. The fluid collected was stored at -20°C until time of assay. Blood samples were taken from the animals by retroorbital bleeding.

Samples were assayed for specific antibody content using the enzyme-linked immunosorbent assay (ELISA). For this, microtiter wells were coated

with a solution containing purified ST preparation (provided by J. Edward Brown) The kinetics of the enzyme-substrate reaction were extrapolated to 100 minutes. For the Shiga toxin assays, the ELISA Amplification System (Bethesda Research Laboratories, Inc.) was used with results measured as OD 490nm/15 minutes. Specific IgG and IgA standards are processed on each plate with the unknown fluids as previously described (7).

As shown in figure 1, all of the doses of ST were immunogenic for an IgA anti-ST response in the secretions obtained. A classic dose response curve was achieved. Although we gave doses of 50 mg to a group of mice, 4 of them died soon after the immunization, therefore, this data is not included. The animals receiving 25 mg all survived, however, for the studies on adjuvanticity of ST, the 10 μ g dose was used to minimize the potential for cytotoxicity of the immunizing dose (see below). The 10 mg dose consistently gave a vigorous IgA anti-ST response in the secretions. By the second week after immunization all of the mice had achieved a detectable response. The response continued to increase and peaked after the third dose. No increase

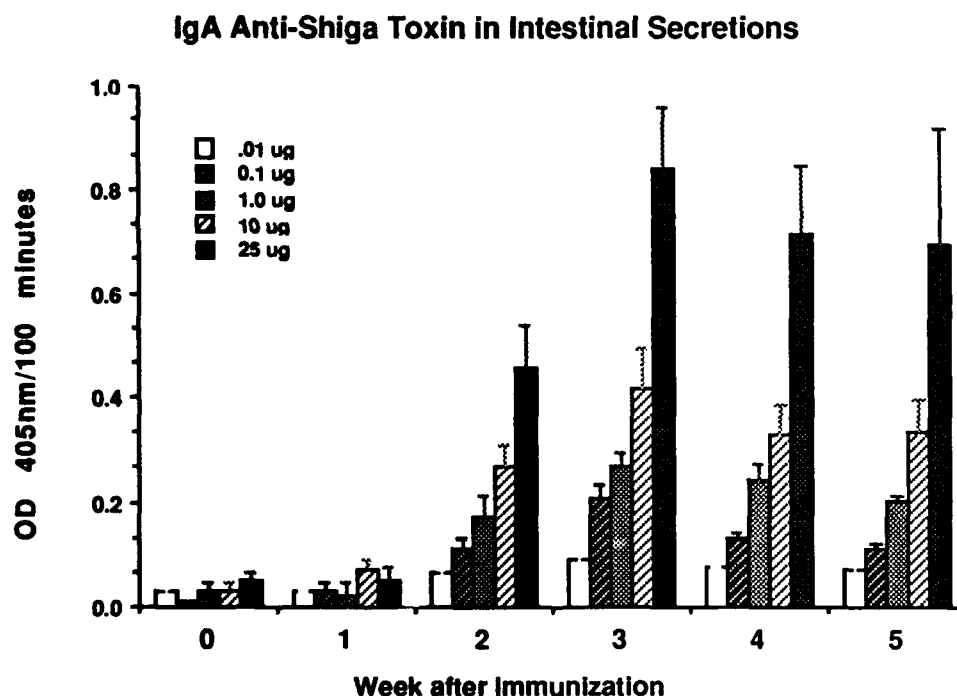


Figure 1. IgA anti-shiga toxin response in intestinal secretions.

was seen after the fourth dose. The response remained at the peak level with no significant decline through the end of the study at 5 weeks. A modest decline was seen with the 25 mg group, however, this was not significant at 5 weeks compared to the 3 week (peak) value.

Similar dose response curves were seen with the IgG and IgM in secretions. The IgG response was as strong or stronger than the secretory IgA response as measured by the ELISA (figure 2). This is different from the responses described by us in the rabbit loop model using crude toxin in previous studies, where relatively trivial amounts of IgG anti-ST were seen in the loop secretions. The difference reflects consistent differences in the mucosal immune systems of mice and rabbits. With virtually all of the immunogens we have used in the rabbit loop model system (CT, ST, Shigella LPS, Salmonella LPS, 2-acetylaminofluorene (AAF), keyhole limpet hemocyanin (KLH), and bovine serum albumin), relatively little antigen-specific IgG has been detected in secretions despite the presence of high-titered antigen-specific IgA. With the mouse model, relatively large amounts of both IgA and IgG have been detected by us.

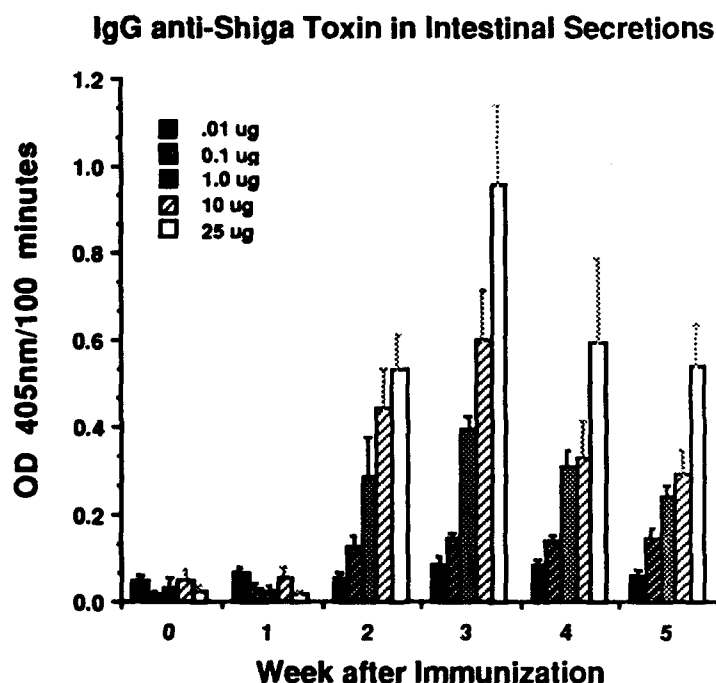


Figure 2. IgG anti-ST in secretions.

Since purified ST is an effective mucosal immunogen which gives a classic dose response curve, we wished to examine whether it was able to provide adjuvant activity like CT, and whether it might even act synergistically with CT to heighten the secretory IgA response to co-administered weak mucosal immunogens. For these studies, we chose to use the standard weak mucosal immunogen KLH. When given orally, or inaintestinally, only trivial amounts of secretory IgA can be detected against KLH, even after multiple immunizations. In our studies, we gave mice 5 mg

of KLH orally alone or with various combinations of CT and ST (each used at 10 mg). Our studies indicate that KLH alone produced only a weak IgA anti-KLH response as expected. When CT was added, a strong local IgA and IgG anti-KLH response consistently resulted. However, ST was totally ineffective at achieving a secretory IgA anti-KLH response. Indeed, the available information suggests that a slight inhibition of the IgA anti-KLH response may occur (figure 3). Whereas ST is one of the strongest mucosal immunogens described thus far, weaker only than CT, ST does not act as a mucosal adjuvant and does not further potentiate the adjuvanticity of CT using the standard KLH system.

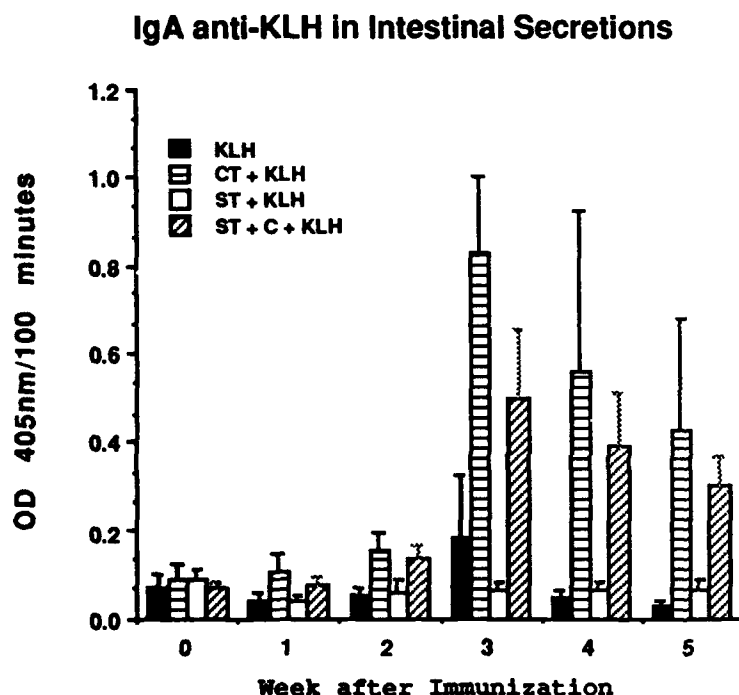


Figure 3. IgA anti-KLH response in intestinal secretions.

E. Biologic Role of Secretory IgA Response to Shigella Antigens.

Two studies on the biologic effect of specific secretory IgA against shigella antigens have been completed at the present time. The first involves study of the ability of secretory IgA anti-Shigella LPS to interfere with the binding and uptake of live, virulent *S. flexneri* in an acutely ligated segment of intestine. The second demonstrates both *in vitro* and *in vivo* the ability of secretory IgA against Shiga toxin to interfere with the cytotoxic effects of that molecule.

1. Role of Secretory IgA to interfere with uptake of live *S. flexneri* by the intestinal epithelium. Based on the results of the studies conducted in our

laboratory on the uptake of *S. flexneri* by follicle-associated epithelium, we inferred that the replication of these bacteria within these epithelial cells inexorably follows their entry into the cytoplasm. Therefore, preventing the initial entry, or decreasing the number of bacteria permitted to enter the epithelial cells should interfere with the damage to the surface epithelium. In this study, two rabbits were immunized with three oral doses of 10^8 live *S. flexneri* 2457-0 given once a week, and allowed to rest for two weeks. However, due to a difficulty in securing a consistently invasive strain (as shown by the Sereny test), we had to wait several weeks before conducting the protection studies. During this time, we gave three more booster doses to these rabbits. When a virulent strain of *S. flexneri* became available, we ligated loops of intestine in these two rabbits and in an unimmunized rabbit as a control. The control rabbit showed a dilated loop with bloody secretions after 18 hours. In marked contrast, there was no evidence of blood in the two immunized rabbits, although there was a watery fluid secretion in these loops. Histologically, the 18 hour loops from the control rabbit showed ulcerations over all the Peyer's patch dome areas with blood in the lumen. The immunized rabbits had rare ulcers with some dome regions completely intact. At the 90 minute time period, there was an 8-10 fold difference in the uptake of the bacteria by the surface epithelium in the immune and nonimmune animals.

We created five groups of rabbits (2 rabbits in each group) to determine the effect of prior immunization on the uptake of the shigella. On day 42, an acute loop study was conducted using 10^8 live *S. flexneri* M4243 injected directly into the isolated loops. The results in table 7 indicated that there was no detectable protection in any of the groups. These results were contradictory to the earlier studies. However, during the preliminary studies, we gave two more immunizing doses, and there was concern about the level of virulence of the strain of shigella used in the final assay. On evaluating the results, we identified two key factors which may have contributed to the lack of protection. First, the dose of virulent M4243 used (2×10^9) was very likely too high to allow demonstration of the protective ability. This many bacteria in the limited surface area of the ligated loop were probably able to overcome the secretory IgA which was present. Unfortunately, when we attempted to use fewer bacteria as a challenge, the amount of uptake of the shigella was too inconsistent to measure. Secondly, the periods examined (90 minutes and 18 hours) may have been far too long. These times were used because our previous study showed that uptake can readily be observed at these times using both ultrastructure and Giemsa staining (21). However, the initial attachment of the shigella occurs much sooner than this (likely prior to 30 minutes).

Therefore, by looking at later time points, we have prejudiced the study in favor of eventual invasion by the shigella.

Table 7. Uptake of Shigella¹ in Rabbits Immunized Orally with Different Shigella Preparations

<u>Antigen for Immunization</u>	<u>Bacteria² Uptake at 90 Minutes</u>			
	<u>Dome Regions</u>		<u>Villus Regions</u>	
<u>Culture Broth</u>	2.79	(0.12)	0.62	(0.08)
<u>Culture Broth</u>	2.27	(0.04)	0.38	(0.03)
<u>S. flexneri 2457-Q</u>	8.35	(0.37)	1.33	(0.19)
<u>S. flexneri 2457-Q</u>	7.36	(0.53)	0.85	(0.24)
<u>S. flexneri M4243A1</u>	3.95	(0.09)	0.61	(0.06)
<u>S. flexneri M4243A1</u>	5.06	(1.04)	0.77	(0.28)
<u>Shigella X16</u>	1.61	(0.01)	0.29	(0.01)
<u>Shigella X16</u>	3.26	(0.35)	0.53	(0.10)
<u>Heat-killed Shigella</u>	4.27	(0.18)	0.91	(0.09)
<u>Heat-killed Shigella</u>	0.76	(0.17)	0.23	(0.08)

1. Animals immunized as described in Table 3. Invasive *S. flexneri* M4243 used to challenge the isolated loops (see text).
2. Uptake expressed as numbers of bacteria completely within host epithelial cells with standard error of means indicated.

In future studies, we believe the use of a RITARD system would allow us to use much more physiologic doses (from the human disease viewpoint) and would give us a better assessment of the role that secretory IgA plays in protection against uptake of shigella.

2. Role of Secretory IgA to Protect against the Cytotoxic Effects of Shiga Toxin. Our studies on the mucosal immune response to Shiga toxin and its functional significance were carried out in collaboration with Dr. J. Edward Brown. His laboratory provided the Shiga toxin preparations and performed the below listed HeLa cell assay. All the rabbit studies, immunizations and protection studies were performed in our laboratory.

Although the role of Shiga toxin in dysentery is unknown, it is cytotoxic to HeLa cells, causes fluid secretion in rabbit intestine and is lethal when injected parenterally to rabbits or mice (28,33).

For the present study, five rabbits were inoculated directly into chronically isolated ileal loops (see Methods section) on the day of surgery (day 0) and on days 7 and 14 postsurgery. They were given 0.5ml of crude shiga toxin preparation (see Methods section) in 4 ml of saline. Intestinal secretions were collected daily and blood samples were collected weekly. A new ELISA for shiga toxin was created for these studies. While the technical

details of the assay are the same as detailed in the Methods section, a four point standard curve was assayed on each plate with the unknown samples. The reciprocal of the dilution giving an O.D. reading between the two lowest values on the standard curve was defined as the titer.

As shown in figure 4, a significant increase in the mean IgG anti-Shiga toxin titer over the day 0 value was detectable in serum by day 7 after the first intraloop immunization. This titer rose after the third dose on day 14 and did not change significantly through the end of the study period on day 30. In contrast to the high titer of IgG anti-Shiga toxin in the serum, only trivial amounts were detected in the loop secretions (figure 3). Thus only a small amount of serum IgG anti-Shiga toxin finds its way into the loop secretions (our previous studies have shown good stability of IgG in the chronically isolated ileal loops).

IgG anti-Shiga toxin in Serum and Secretions

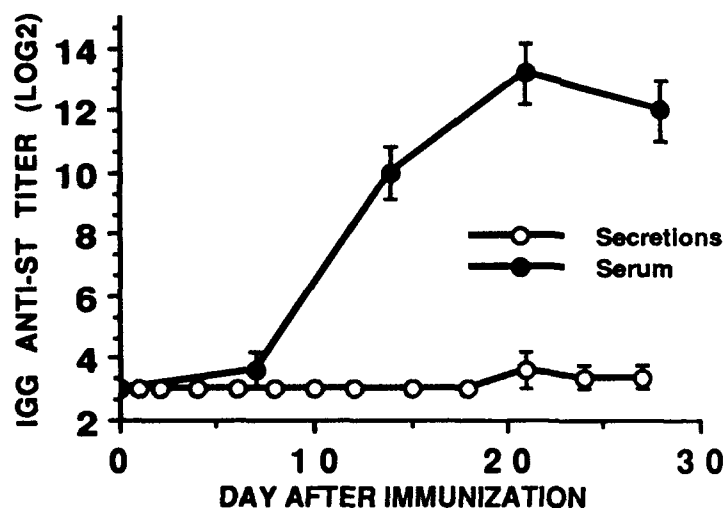


Figure 4. IgG anti-Shiga toxin in animals immunized orally with crude Shiga toxin.

The IgA anti-Shiga toxin titer in the serum of these rabbits was lower than the IgA titer in secretions (figure 4). A significant ($P < .01$) increase in IgA anti-Shiga toxin titer of the serum over the day 0 values was seen by day 14. In the loop secretions, as early as day 2, a weak but significant ($P < .05$) increase in the IgA anti-Shiga toxin titer was seen (figure 5). The content of

IgA anti-Shiga toxin declined on the day after the third intraloop dose (day 14), but had another striking increase three days later. After this peak on day 18, the mean IgA anti-Shiga toxin titer slowly declined, although it never dropped below the level of activity seen after the second intraloop dose on day 7. It is possible that the slight decline in IgA titer seen the day following each booster immunization reflects the presence of free toxin in the loop which binds to the specific IgA. Alternatively, Shiga toxin may interfere with local antibody synthesis or secretion of IgA into the gut lumen.

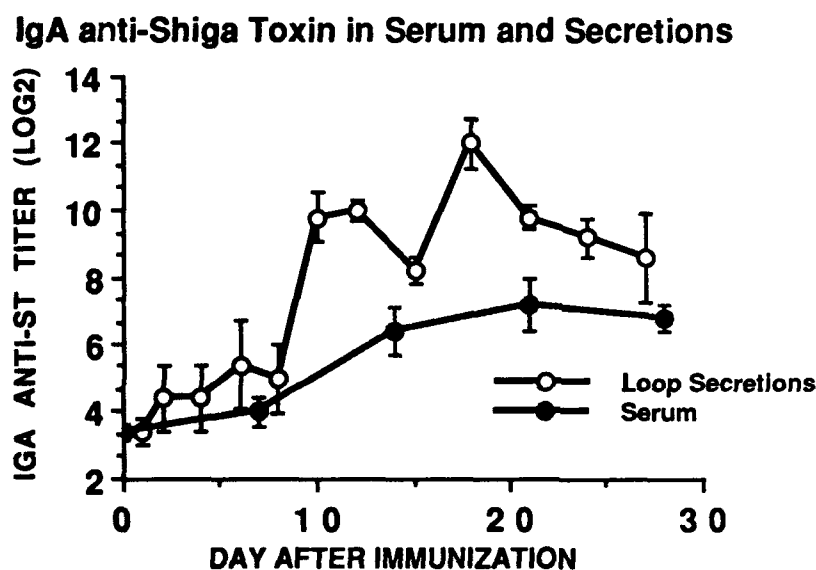


Figure 5. IgA anti-Shiga toxin in animals immunized orally with crude Shiga toxin.

To assess the *in vitro* Shiga toxin neutralizing activity of intestinal loop secretions, a HeLa cell assay was performed. For this assay, HeLa cell monolayers were grown in 96 well microtiter plates and a standard crude toxin lysate of *S. dysenteriae* was incubated with serial dilutions of loop fluids for 30 minutes at room temperature. This mixture was placed onto the HeLa cell monolayer and allowed to incubate overnight at room temperature. The monolayers were then stained with crystal violet and the O.D. 620nm was determined for each well. The dye remaining in each well correlates with the percentage of cells remaining adherent to the microtiter dishes (28). O.D. 620 nm of wells containing the standard toxin alone were averaged and that value plus two standard deviations was defined as the end point titer of loop fluids

for neutralization of the cytotoxicity of the toxin preparation. All loop fluids which gave an O.D. 620nm in the assay greater than this value were scored as positive.

The Mean Shiga toxin neutralizing activity in the HeLa cell assay is depicted in figure 6. The curve in figure 6 shows the same basic triphasic response as the IgA anti-Shiga toxin in loop secretions from figure 4. The correlation coefficient of the mean IgA activity in secretions with the mean toxin neutralization titer was .928 while the correlation of the IgG level in secretions with the mean toxin neutralization titer was only .116.

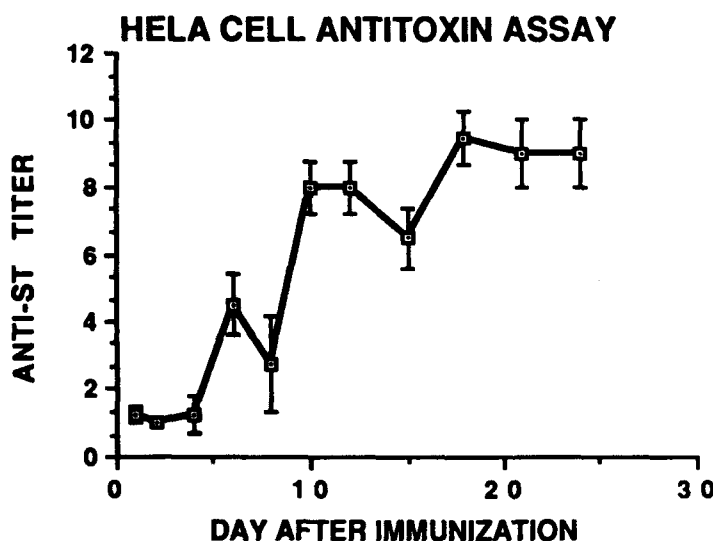


Figure 6. Anti-Shiga toxin activity *in vitro* of loop secretions from animals given oral immunization with crude Shiga toxin.

To assess the *in vivo* Shiga toxin neutralizing activity of intestinal loop secretions, an acute loop protection model was devised. Pooled loop secretions from rabbits with high titer IgA anti-Shiga toxin activity as determined by ELISA were diluted 1:2 in saline and mixed with an equal volume of a 1:256 dilution of crude toxin. This was injected into 5 cm isolated segments of ileum in unimmunized rabbits. This dose of toxin was chosen as it consistently elicited fluid accumulation when given to acutely ligated loops. As controls, toxin was mixed with secretions from nonimmune animals or saline and injected into other loops in the same rabbit. After 18

hours, the animals were sacrificed and the volume of fluid in each segment was measured.

Pooled loop secretions from animals immunized with Shiga toxin reduced toxin-induced fluid accumulation in the acutely ligated rabbit intestine. Secretions with no detectable IgA or IgG-anti-Shiga toxin by ELISA had no inhibitory effect on the Shiga toxin-induced fluid production by rabbit intestine (figure 7). The heterogeneity shown by the standard errors of the means reflects the differential response of the genetically diverse outbred rabbits used in these studies. Even with this degree of heterogeneity, the difference between the fluid production in loops protected with immune secretions and those given nonimmune secretions was highly significant ($P < .01$).

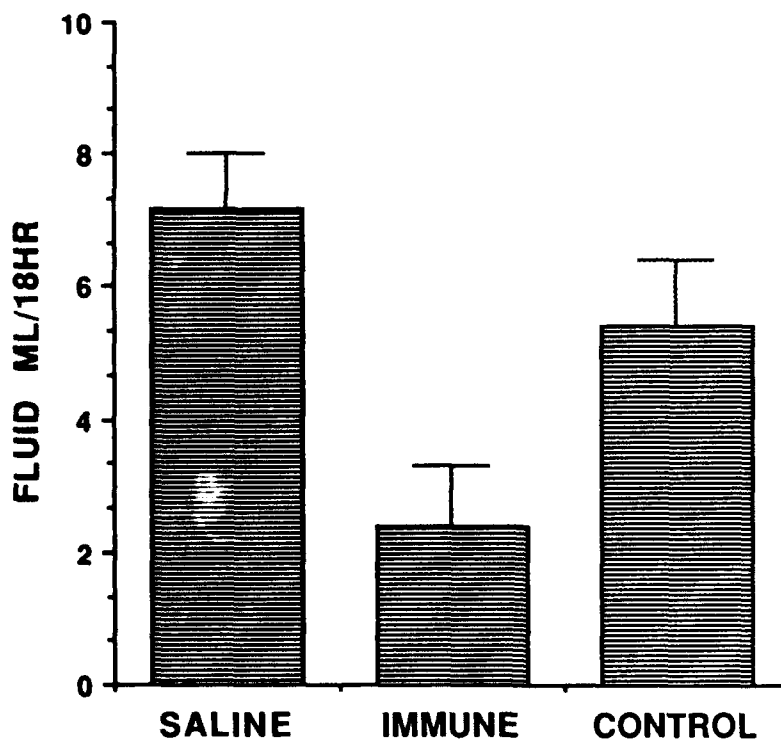


Figure 7. Anti-Shiga toxin activity *in vivo* of loop secretions from animals given oral immunization with crude Shiga toxin.

These findings indicate that a strong secretory IgA mucosal immune response can be elicited to Shiga toxin and suggest that such antibodies could interfere with the toxic effects both *in vitro* and *in vivo*.

F. Role of *E. coli* heat-labile toxin B subunit as a mucosal adjuvant for *Shigella* LPS.

Because of the lack of effect of ST as an adjuvant (see above), we wished to examine the effect of the cloned B-subunit of the heat labile toxin from *E. coli* (LT-B) as an adjuvant for relevant shigella antigens (34). We took advantage of previous studies by our laboratory that had demonstrated only a weak IgA response resulting from intra-Thiry-Vella loop administration of four doses of 400 ug of purified *S. flexneri* LPS. LPS-LTB was administered into chronically isolated ileal loops on days 0, 7, 14, and 21. Peripheral blood was drawn on a weekly basis, and ileal loop secretions were collected daily. The samples were stored at -20C until assayed. The control group for these experiments consisted of four rabbits receiving only LPS (400 ug) on the same dosing and bleeding schedule. A second control group consisted of four rabbits given 400 ug of LPS plus the mass equivalent amount of LT-B present in the conjugate (13%, or 52ug). This "admixed" group was also treated on the same dosing and bleeding schedule as the previous two groups.

Once each rabbit was carried through day 48, it was sacrificed and 50 or more ml of serum was removed via heart puncture. All ileal loop fluids were serially diluted in log₂ dilution from 1:16 to 1:1024 (or log₂ = 4 to 10) and scored for titer of IgA anti-LPS. Titers less than 4 were excluded and scored for titer of IgA anti-LPS. Titers less than 4 were excluded and scored as a negative responder. Each animal was separately scored for anti-CTB response (immunologically identical to LT-B). The mean log₂ values were calculated for the responding animals in each group and comparisons were made without the use of statistical analysis due to an obvious lack of difference between treatment groups.

As shown in figure 8, a substantial amount of radioactivity eluted in the void volume, representing LT-B bound to the residual protein A in the LPS preparation. Assuming that the original mass of LPS was retained, the final preparation was judged to contain approximately 13% LT-B by weight (calculated from the specific activity of the ¹²⁵I-LT-B). This preparation demonstrated both the LPS and CTB epitopes using known positive anti-LPS and anti-CTB antiserum when tested by ELISA. In addition, the conjugate bound to GM1 ganglioside coated to microtiter plates, and retained the ability to express the LPS epitope (suggesting covalent attachment without loss of GM1 binding capacity).

S-300 Elution of LPS-LTB-5

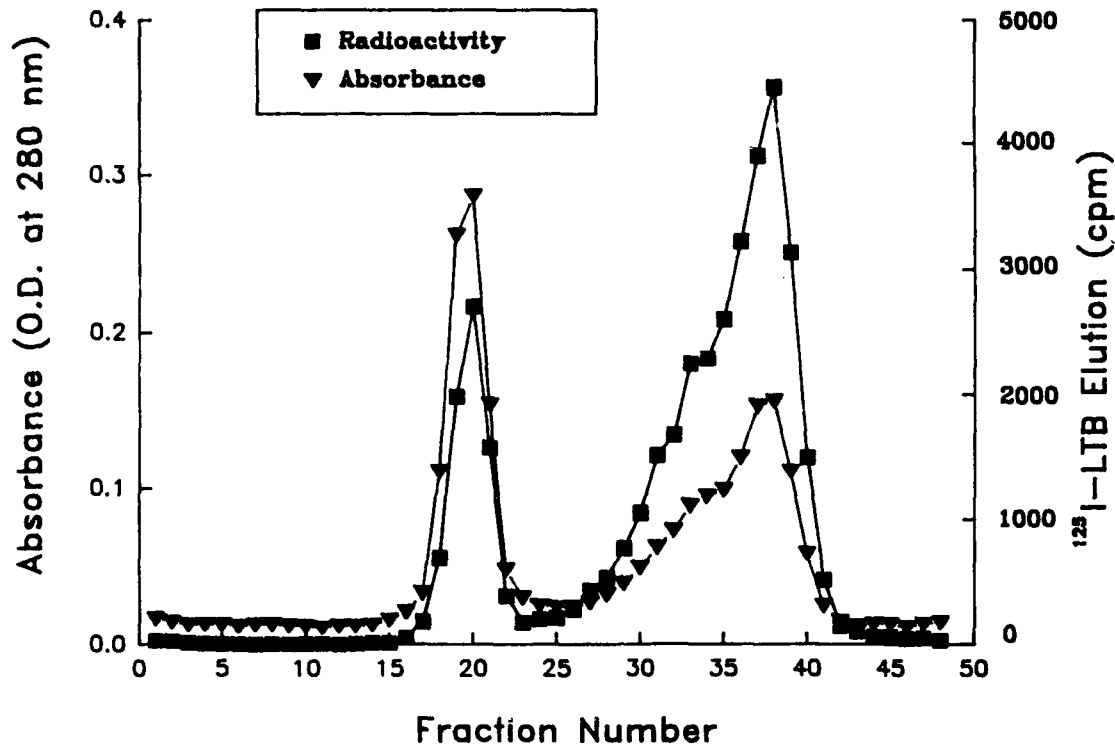


Figure 8. Elution of LPS-LTB-5 from S-300 column.

Chronically isolated ileal loops were installed in three groups containing four rabbits each. The conjugate preparations or control fluids were administered as discussed above. Group 1 received LPS alone, group 2 received LPS-LTB-5 conjugate described above, and group 3 received equal quantities of LPS + LT-B "admixed". As shown in table 8, each group demonstrated roughly equal amounts of anti-LPS binding, while the groups receiving the LT-B, whether conjugated or not, mounted an extremely strong anti-CT-B response.

Table 8. Anti-LPS and anti-CTB titer in Secretions from Rabbits given Oral LPS-LT-B preparations.

Protocol	Rabbit	anti-LPS	Anti-CTB
LPS Alone	M-385	4	<4
LPS Alone	M-386	<4	<4
LPS Alone	M-387	5	<4
LPS Alone	M-388	<4	<4
LPS- LTB-5	M-389	<4	8
LPS- LTB-5	M-390	6	9
LPS- LTB-5	M-391	<4	>10
LPS- LTB-5	M-392	5	<4
LPS + LTB	M-393	4	>10
LPS + LTB	M-394	5	>10
LPS + LTB	M-395	5	8
LPS + LTB	M-396	5	9

* Results expressed as log2.

As is evident from the data in table 8, the presence of covalent binding of LPS to LT-B moieties did not appreciably increase the mucosal immune response to LPS. All preparations containing LT-B elicited a strong secretory IgA anti-LT-B response in all but one rabbit (7/8). Further, there is a suggestion that the group 3 rabbits (LPS + LTB) has a more consistent mucosal immune response against the LPS than the other groups. This may indicate that the animals have been effectively primed and may be able to mount a mucosal memory response against a challenge by intact microorganisms. Although not central to the present studies, the fact that there was one nonresponder to the highly potent mucosal immunogen LT-B

emphasizes the extreme polymorphism that exists in the mucosal immune response in outbred animals. Thus, one must predict the same type of extreme heterogeneity when faced with vaccinating a human population. While the majority of individuals will respond to mucosal immunogens such as CT, ST or LT-B, we will doubtless encounter individuals who will prove to be non- or pauciresponders.

CONCLUSIONS

These studies demonstrate several major features which are important if major efforts are to be made to use mucosal vaccines to effectively protect humans against enteric diseases. First, a major route of uptake of the intact shigella is by the M cells which lie in the follicle-associated epithelium overlying lymphoid follicles throughout the gastrointestinal tract. When the *S. flexneri* taken up are virulent, they proliferate within these cells eventually producing ulceration at these sites. The presence of many such follicles in the colon and the terminal ileum may explain the frequency of focal ulcerations at these sites in clinical dysentery. Ideally, one would wish to interfere with the initial adherence of the shigella to the surface epithelium to prevent clinical disease. Since our previous studies have shown a strong secretory IgA memory response in intestinal secretions following oral immunization with live, attenuated strains, we sought to use intact rabbits as a model system to evaluate the ability of secretory IgA to interfere with the uptake and ulcerations which occur. For these studies, we performed investigations with rabbits given several oral doses of live, attenuated strains of shigella. However, in order to obtain reproducible uptake and ulcerations, large doses of shigella were required. This, of course, contrasts with the human situation where only small numbers of shigella can produce clinical dysentery. Therefore, we were not able to demonstrate protection with the vaccinated animals.

We have established the cellular basis for the mucosal memory response. By using cell cultures from Peyer's patches, mesenteric lymph nodes, spleen and peripheral blood from animals given various immunization regimens with attenuated strains of *S. flexneri* we found that by the third day following oral rechallenge, memory B cells are present within the Peyer's patches and mesenteric lymph nodes. In unimmunized animals, virtually no antigen-specific response is found on these days after oral challenge. Further, by this time, the cells are already committed to synthesize the IgA isotype. Our most recent studies show that the population of helper T cells which are present in GALT play an integral role in stimulating peripheral blood and spleen B lymphocytes (from primed animals) to mature into IgA producing plasma cells. Although it has been known for some time that B lymphoblasts

circulate through these sites, it was not known that T helper cells from GALT are the key element to switch on the production of IgA against antigens which had been seen only by the mucosal lymphoid system. The information from these studies could have practical importance for testing new vaccine preparations for enteric infections.

The mouse model system for evaluating the mucosal immune response to shigella antigens has been expanded to study the role of Shiga toxin. The data from these studies indicate that a strong secretory IgA response to Shiga toxin is elicited and that the level of the response seems to be dose dependent. We strongly encourage the use of the mouse lavage system for future studies because many monoclonal reagents and inbred strains are now available which allows us to dissect more precisely the cellular basis of the secretory immune response to shigella antigens. This model would have been very advantageous in trying to determine the subtypes of T cells involved in the mucosal memory process. In the rabbit studies discussed above, this type of study was not possible.

The heterogeneity of the secretory IgA response against Shiga toxin has been documented. These studies have demonstrated that a strong secretory IgA response can be elicited to Shiga toxin and that this response can protect both *in vivo* and *in vitro* against the toxic effects of that molecule. Although these findings have also suggested Shiga toxin as a potential mucosal adjuvant which may be used to augment the mucosal immune response to other antigens, our more recent studies failed to confirm this role for Shiga toxin. To date, we find that only cholera toxin has given such a strong secretory IgA adjuvant effect in our rabbit model system. Our efforts to use *E. coli* LT-B as a mucosal adjuvant for shigella LPS gave only weak results. Thus, we find, at the present time, only cholera toxin has been a reliable mucosal adjuvant.

These studies have provided new information on the cellular basis of the mucosal memory response to shigella antigens. While this work has obvious practical implications to shigella many of the findings, especially the possible role of Shiga toxin as a mucosal adjuvant, have potential application to other enteropathogenic infections is also provides basic details about the secretory IgA memory response and its functional significance. Information about the cellular basis of the mucosal memory response allows us to test potential vaccine strains against a wide variety of infectious agents in a fraction of the time and expense previously required by the reliable, but slow and costly Thiry-Vella loop technique. Lastly, we have established the mouse model for mucosal immunity for shigella antigens. Our former Thiry-Vella loop model system worked well, but was a highly specialized model which was difficult for many laboratories to reproduce and use well. The mouse lavage technique

first proposed by Elson has proven to be a much simpler technique which many laboratories can reproduce. The inbred mice provide both advantages and disadvantages for future studies. The major advantages relate to the ready availability of biologic reagents to specific cell subsets. One disadvantage which should be remembered, is that the inbred population will not give a good overall indication of the breadth of the outbred human population's response to a vaccine preparation. For this type of information, subsequent investigations using outbred animals (such as the rabbit Thiry-Vella loop model) is recommended.

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